INVESTIGATIONS IN FISH CONTROL

35. Toxicology of Thiodan in Several Fish and Aquatic Invertebrates

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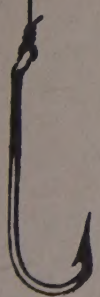

United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

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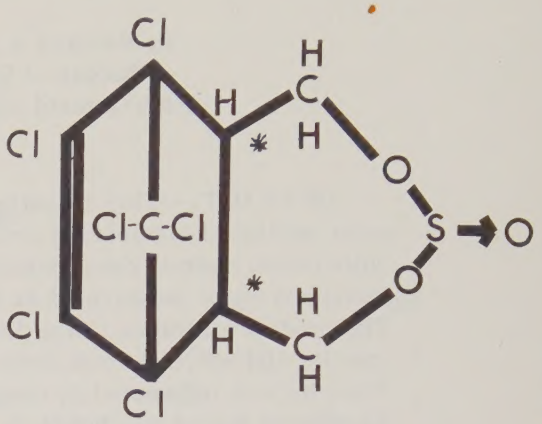
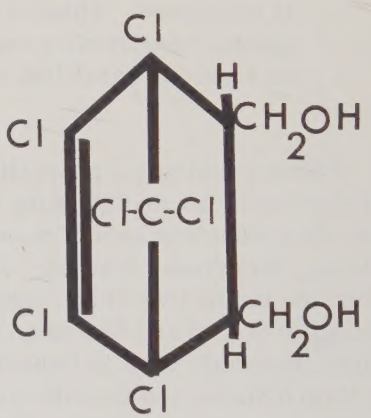
MATERIALS

Thiodan is the registered tradename of a chlorinated fused-ring heterocyclic compound developed by Farbwerke Hoechst A. G., Frankfurt, Germany, as an insecticide (F.M.C. Corporation, 1964). The Food Machinery and Chemical Corporation (F.M.C.), Niagara Chemical Division, Middleport, N.Y., is licensed to manufacture, use, and sell Thiodan in the United States. The American common name of Thiodan is endosulfan (6,7,8,9,10,10-hexochloro 1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide).

The empirical formula is $C_9H_6Cl_6O_3S$ and its structure is as follows (F.M.C. Corporation, 1958a and 1964):



According to the manufacturer, the technical Thiodan used in this study is 96.4 percent pure. It contains A and B isomers which melt in the ranges of 108° to 109° C., and 206° to 208° C., respectively (Lindquist and Dahm, 1957). The acid hydrolysis of either isomer yields only one isomer of Thiodan alcohol which has the structure:



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The F.M.C. Corporation supplied approximately 17 milligrams of carbon-14

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Desired concentrations of Thiodan were prepared by adding aliquots of 1-part-per-thousand stock solutions of technical Thiodan in ethanol to the water. The concentrations were based on active ingredient. Ethanol was added to controls in an amount equal to the largest aliquot of stock used in the test series. A minimum of aeration was applied to all of the solutions tested against fish to maintain an oxygen concentration of not less than 5 ppm. Ten fish and 10 or 20 invertebrates were used to measure toxicity, and the trials were replicated 1 to 4 times. The loadings of fish in test vessels were approximately 0.5 to 1.0 gram per liter.

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Observations on the toxicity of Thiodan were made 24, 48, 72, 96, and 120 hours after the experimental animals were first exposed. Individuals showing no respiratory movement and no response to a tactile stimulus were recorded as dead and were removed.

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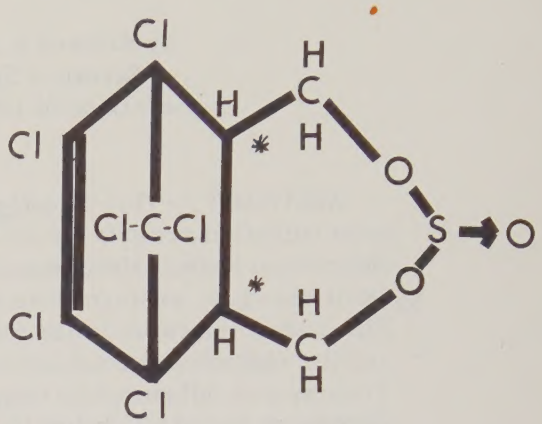

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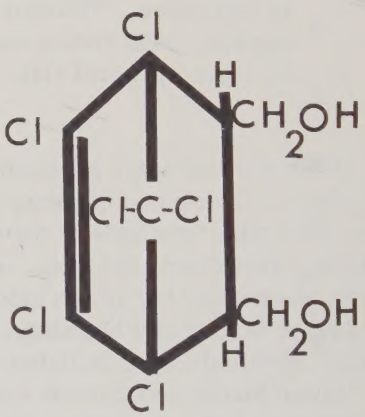

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INVESTIGATIONS IN FISH CONTROL

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La Crosse, Wisconsin 54602

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United States Department of the Interior, Walter J. Hickel, *Secretary*
Leslie L. Glasgow, *Assistant Secretary for Fish and Wildlife, Parks, and Marine Resources*
Fish and Wildlife Service, Charles H. Meacham, *Commissioner*
Bureau of Sport Fisheries and Wildlife, John S. Gottschalk, *Director*
Washington, D.C. · January 1970

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TOXICOLOGY OF THIODAN IN SEVERAL FISH AND AQUATIC INVERTEBRATES

By Richard A. Schoettger, Fishery Biologist
Bureau of Sport Fisheries and Wildlife
Fish Control Laboratory, La Crosse, Wisconsin

ABSTRACT.--This investigation provides toxicological data on Thiodan to meet published criteria for evaluating chemicals for fish control. Thiodan, a chlorinated hydrocarbon insecticide, was tested against rainbow trout and their fertilized eggs, western white suckers, Daphnia magna, and damselfly naiads. The median tolerance limits for trout and suckers ranged between 0.3 and 8.1 ppb and the fish were at least seven times more susceptible than the invertebrates. Toxicity was influenced by temperature, length of exposure, and alkaline pH. Exposures for up to 2 hours to 50 ppm of Thiodan were not toxic to fertilized trout eggs. The deposition and metabolism of Thiodan residues in western white suckers, northern creek chubs, and goldfish were traced with the aid of carbon-14 labeled Thiodan, and chemical analyses of Thiodan in tissues. Residues occurred in the skin and muscles of fish exposed to acute and to multiple subacute concentrations. The death of fish which were poisoned subacutely was correlated with size and the lipid content of muscle. The residues in the various tissues seemed to be associated with the method of treatment. A water-soluble metabolite of Thiodan in the bile of treated fish appeared to be a glucosiduronic acid conjugate of Thiodan alcohol. A possible metabolic pathway for Thiodan degradation is discussed. Thiodan appears to have little value as a selective piscicide against rough fishes such as carp or suckers, but under certain conditions it may be a good general fish toxicant.

Fishery biologists generally turn to the use of chemicals for manipulating fish populations when other methods of management such as seining, drawdown, draining, or biological controls are ineffective or too expensive. According to Stroud and Massman (1963) the principal chemicals used in fishery reclamations in the United States and Canada are the insecticides rotenone and toxaphene. They reported that another insecticide, Thiodan,[1/] was applied experimentally to four small lakes in Canada. The trials indicated that it is highly toxic to fish, detoxifies more rapidly than toxaphene, and is less expensive than rotenone, but may be detrimental to plankton and bottom fauna.

Thiodan and toxaphene are classed chemically with the organochlorine or chlorinated camphene insecticides, which include aldrin, dieldrin, and endrin. It is generally recognized that this group of chemicals is relatively persistent in the environment. For example, toxaphene-treated waters may remain toxic to fish for 2 or 3 years (Tanner and Hayes, 1955; Hooper and Grzenda, 1955); residues of toxaphene may persist in fish tissues and in other parts of the ecosystem (Kallman, Cope, and

1/ The term Thiodan (the trade name for endosulfan) is used in this paper because it is in common usage.

This paper is based on a dissertation submitted to the Faculty of the Zoology Department of Colorado State University in 1966, in partial fulfillment of the requirements for the degree of doctor of philosophy. Financial assistance from the National Institutes of Health is gratefully acknowledged.

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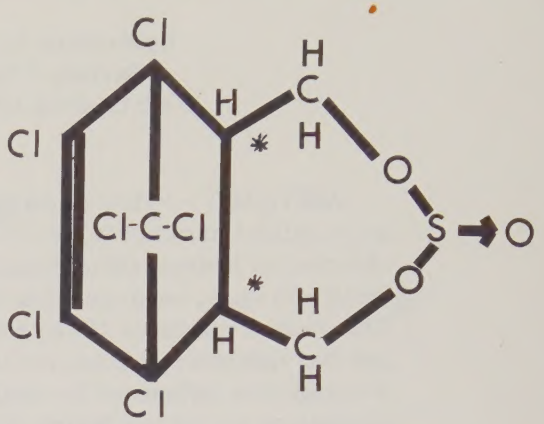

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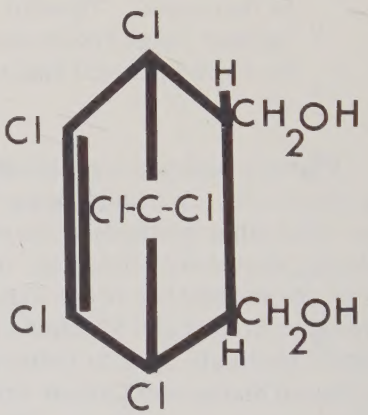

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Table 1:--Species, sizes, and sources of experimental animals.

Species	Size range Length (inches)	Weight (grams)	Source
Rainbow trout, Salmo gairdneri	1.6-2.2	1.0-1.8	SFH[1], Bellvue, Colo.
Western white suckers, Catastomus commersoni	1.8-2.6	0.9-2.5	Cache la Poudre River, Fort Collins, Colo.
Do.	5.3-8.2	40.1-70.0	
Northern creek chubs, Semotilus atromaculatus	6.7[2]	48[2]	
Goldfish, Carassius auratus	6.7-10.9	96-337	NFH, Genoa, Wis.
Rainbow trout (fertilized eggs)	---	---	NFH, Manchester, Ia.
Water fleas, Daphnia magna	---	---	Laboratory cultures at Colorado State Univ., Fort Collins, Colo.
Damselfly naiads, Ischura sp.	0.25-0.50	---	From beneath the ice of a pond near Fort Collins, Colo.

1/ State fish hatchery.
2/ Mean.
3/ National fish hatchery.

The TLm's for 72, 96, and 120 hours were interpolated from averages of the replicate bioassays by methods of the American Public Health Association (1960).

Eggs of rainbow trout were fertilized at the National Fish Hatchery, Manchester, Iowa, and transported to the laboratory. Twenty-five hours later they were divided into groups of 100 each and dipped into solutions containing 10, 100, 1,000, 10,000, or 50,000 ppb of Thiodan for 30 or 120 minutes. Test solutions were prepared with technical Thiodan dissolved in acetone, and control solutions with a corresponding concentration of acetone alone. After dipping, the eggs were placed in a Heath Incubator at 12° C. for hatching. At 24-hour intervals the eggs were observed for mortality, and 2 or 3 specimens were collected randomly for microscopic examination. The samples were prepared for the examination, and stages of development were identified as described by Knight (1963).

Solutions for testing water quality effects on toxicity

Salt solutions were made to a hardness of 500 ppm, as calcium carbonate, by adding calcium chloride, calcium sulfate, magnesium chloride, or magnesium sulfate to 3 liters of tap water in 1-gallon glass jars. Thiodan dissolved in ethanol was added to make a concentration of 20 ppb. Controls were salt solutions without the toxicant, solutions with Thiodan, but no salts, and vessels with only tap water.

Effects of pH on toxicity were measured in solutions of pH 6.4, 7.4, 8.4, and 9.4. The pH was adjusted by adding potassium hydroxide and/or acetic acid to 3 liters of tap water in 1-gallon glass jars. A concentration of 20 ppb of Thiodan was added to each jar. Control solutions had pH values appropriate for a particular pH test, but were without Thiodan.

Following preparation of experimental media, the glass jars were sealed and the contents aged at 19° C. $\pm$ 2° to determine degradation time. Salt solutions were aged up to 436 hours, and solutions of different pH for up to 96 hours. Then they were aerated and bioassayed for 24 hours with 10 suckers with an average length of 2 inches and weight of 1.3 grams.

The comparative differences in toxicities of the solutions were analyzed statistically by the exact test for 2 x 2 tables (Goulden, 1956).

Deposition and metabolism of Thiodan

Radioactive Thiodan--Western white suckers were bioassayed in 1-gallon glass jars containing 1.5 or 3.0 liters of tap water at 19° C. One sucker was placed in each jar per 1.5 liters of solution. The fish had an average length of 5.3 inches and weighed 40.1 grams. Radioactive Thiodan was dissolved in ethanol, and a stock was prepared as in the toxicity experiments. Portions of this stock were added to the jars to give concentrations of 20 and 80 ppb of labeled compound. At the time of these experiments, the exact purity of the labeled Thiodan was unknown. Forman et al. (1960) later reported that it contained 85- to 93-percent Thiodan, but the impurities were not radioactive. Thus, the amount of pure Thiodan in each jar was slightly less than 20 or 80 ppb.

The fish exposed to a concentration of 80 ppb were removed for analysis at the time of death. Those treated with 20 ppb were killed with chloroform at intervals between 1 and 2 hours of exposure. Samples of gill filament, heart, liver, kidney, skin, muscle, gut (with and without feces), brain, and blood were collected. Blood was obtained by cardiac puncture immediately after death. The samples, excluding blood, were rinsed briefly in distilled water, placed in tared vials, and dried at 90° to 100° C. to constant weight. They were digested in 3.0 ml of 2-percent potassium hydroxide. A 0.5-ml aliquot of each digest was placed on a tared stainless steel planchet of approximately 20 cm and diluted with deionized water. A homogeneous distribution of the material on the planchet was promoted by stirring in a small quantity of laboratory detergent which reduced surface tension. This also reduced contraction of the samples on the planchets while they dried to constant weight. The samples were then ready for measurement of radioactivity.

Determinations of radioactivity were made with an internal proportional counter and the counts corrected for background and counter efficiency. Characteristics of the counter were reported by Nader, Hagee, and Setter (1954). The 0.95 counting error was computed for each sample according to the nomograms of Jarret (1946). Concentrations of Thiodan greater than the counting error were considered significantly different from zero. Untreated control fish were also counted, but none contained statistically significant radioactivity.

Radioactive Thiodan was used to determine whether and how it may be metabolized in fish. Large northern creek chubs and western white suckers were exposed to 40 ppb of labeled Thiodan at 21° C. using methods and materials described in the radiation studies. The fish were killed with chloroform after 3 or 5 hours of exposure.

Samples of chub liver, kidney, blood, and brain were dried to constant weight at 90° C., macerated, and extracted in absolute ethanol. The ethanol was evaporated almost to dryness at room temperature, and the residues were washed with alternate rinses of benzene and deionized water into 125-ml separatory funnels. The mixtures were agitated and allowed to separate for 1 to 12 hours, depending on persistence of emulsions. The emulsions persisted with all samples, except brain. Aliquots of benzene or water fractions were placed on planchets and dried for measurements of radioactivity. Small amounts of persistent emulsions were included with the water fraction. The counts were corrected for background, geometry and backscatter, but not absorption since the amount of lipid per planchet was usually less than 0.2 mg.

The tissues which had been extracted with alcohol were prepared for analyses of radioactivity as described earlier for potassium hydroxide digests of sucker tissue.

The gallbladder bile of chubs and suckers was dried to constant weight at 65° C. and rehydrated in 30-percent ethanol in water. Aliquots of the rehydrated bile were washed with benzene and water into separatory funnels, separated, and analyzed for radioactivity like the alcoholic extracts of chub tissues. Additional aliquots

were diluted to 10 ml with deionized water. Approximately 1 ml of 0.1M sodium acetate and 3.0 ml of 0.2M acetic acid were used to buffer the bile to pH 4.5. The buffered mixtures were incubated for 24 hours at 37° C. with 0.5 ml (2,500 Fishman units) of beta-glucuronidase,[2/] a procedure similar to that described by Talalay (1963). The samples were raised to pH 7.5, then extracted with benzene, and the levels of benzene- and water-soluble radioactive substances were determined.

After approximately a 6-month delay in the research, a check was made of the molecular integrity of the stock supply of radioactive Thiodan. The results of paper chromatographic analyses, methods according to Mills (1959), indicated that the labeled chemical had degraded to what appeared to be Thiodan alcohol. Experiments with radioactive Thiodan were discontinued.

Nonradioactive Thiodan-- Further investigations on the deposition and metabolism of Thiodan in fish were conducted at the Fish Control Laboratory, La Crosse, Wis. Goldfish were maintained in flowing well water at 12° C. and fasted for 24 hours before the tests. An individual was placed in each of 10 glass jars containing 15 liters of reconstituted water at 12° C. The water was reconstituted by adding various salts to deionized water, methods according to Lennon and Walker (1964). Stock solutions of technical Thiodan were prepared in acetone and an aliquot containing 0.105 mg. of toxicant was added to each test solution to give a concentration of 7.0 ppb. Control fish were exposed to similar quantities of acetone. The living test and control fish were transferred daily into new test solutions. The solutions were aerated periodically to insure adequate oxygen.

The goldfish experiment was terminated after 21 days. All of the fish, except two, died during the tests and were removed for tissue analyses. The two survivors were killed by overanesthetization in MS-222. The gill filaments, gonads, livers, spleens, hearts, kidneys, guts (including feces), skin, muscle, brains, peritoneal fat (from two specimens), scales, and fins were dissected from the dead fish. Heparinized samples of blood were collected by cardiac puncture, and the gallbladder was removed from the liver.

According to preliminary tests using analytical methods which are outlined later, I found it necessary to combine tissues, except muscle, from several fish. Samples of approximately 10 to 50 grams were needed for the analyses. The gills from the first five fish which died were combined into one sample, those from the last three and the two survivors into another. Samples of the skin, liver, gut and content, gonad, and two composite samples, one of the heart, kidney, spleen, and blood and another of the scales and fins were prepared like the gill. All of the brains were pooled into one sample. Peritoneal fat was dissected from two fish and pooled. The various samples were weighed and combined immediately after dissection. They were weighed again, after drying to constant weight at 70° C., to determine water content. The gallbladder bile was measured volumetrically and set aside for other analyses. The dried tissues were extracted with petroleum ether for 5 to 6 hours in a soxhlet apparatus. The lipid content was determined by allowing the petroleum ether to evaporate and weighing the residue. They were then redissolved in the same solvent.

The extracts were analyzed for Thiodan by the colorimetric procedure described by Maitlen et al. (1963). He observed that captan, chlordan, heptachlor, and ovex caused some interference in the method, but 45 pesticides and several chlorinated solvents did not. The method is applicable for 5 to 50 ug of Thiodan and also measures Thiodan alcohol. The analyses were made on a Beckman Model DB spectrophotometer.

Before Thiodan can be analyzed colorimetrically, it must be separated (cleaned up) from lipids in ether extracts. Cleanup was

2/ Ketodase. Warner-Chilcott, Morris Plains, N. J.

accomplished by acetonitrile-petroleum ether partition, and by chromatography on a 60/100 mesh, activated Florisil-carbon column described by Murphy and Barthel (1960).

Efficiency of the cleanup technique was determined by running Thiodan standards, or known amounts of Thiodan added to fish fat through the procedure. The columns were eluted with 8-percent diethylether in petroleum ether. The overall recovery averaged 56.3 percent and varied approximately 3 percent. The average recovery was used to correct measurements of Thiodan in fish tissues. Maitlen et al. (1963) obtained recoveries of approximately 77 percent, and Moats (1963) indicated that only one isomer of Thiodan is eluted from Florisil. Thiodan alcohol was recovered completely after acetonitrile partition, but, as was also reported by Moats (1963), none could be eluted from the Florisil column.

In spite of cleanup, low levels of lipids persisted in Thiodan-fish fat standards and in the tissue samples, particularly those from muscle. The lipids did not appear to significantly alter absorbance or wavelength. Analyses of standards containing even greater lipid levels and known amounts of Thiodan supported the observations.

The bile samples were composed of bile from two fish which died on the same day, or successive days. For example, the bile of fish which died on the fourth and fifth days of treatment were combined into one sample, that from individuals dying on the seventh and eighth days formed another, and so forth. The samples were extracted with petroleum ether in a separatory funnel before and after incubation with beta-glucuronidase, as in the radiation experiments. The extracts were analyzed colorimetrically for Thiodan.

RESULTS AND DISCUSSION

Toxicity

Fish--The toxicity of Thiodan to rainbow trout and western white suckers is influenced by temperature and expsoure. The 24- to 120-hour TLm's for trout at 1.5° and 10° C. range from 5.9 to 0.7 ppb and 2.1 to 0.3 ppb respectively (table 2). The TLm ranges for suckers at 10° and 19° are 8.1 to 2.5 ppb and 6.6 to 2.8 ppb (table 2). The results show that in general the toxicity of Thiodan to these species of fish increases with warmer temperatures and longer exposures.

The relation between toxicity and temperature and exposure is shown graphically in figure 1. In general, toxicity increases significantly up to approximately 72 or 96 hours and suggests that the toxicity is associated with accumulations of Thiodan in excess of the amounts that may be metabolized or stored by the fish. Relatively small changes in toxicity occurred with additional exposure (96 and 120 hours), with the exception of suckers at 10° C. The plotted line for suckers at 10° is linear whereas that for trout is curvilinear. The latter species may be able to metabolize Thiodan more efficiently at 10° than the former, though not in equivalent amounts since suckers were more resistant.

The nonlinearity of several of the curves in figure 1 may also be related to the amount of toxicant available for uptake by fish in static tests such as these. Holden (1962) showed, for example, that within 10 hours 80 to 90 percent of the radioactive DDT in static tests was taken up by the fish, detritus, or on the sides of the containers. It is possible that constant-flow tests, or tests where the toxicant is periodically renewed would reveal greater toxicity over longer exposures.

Extrapolations of the 24- and 48-hour regressions used to determine TLm's for trout and suckers indicate that, with one exception, there is less effect of temperature and exposure on the concentrations necessary for 100-percent mortality than on the TLm's. The 100-percent lethal levels for trout are approximately 3 to 5 ppb and 8 to 10 ppb for suckers. The 24-hour concentration of 9.5 ppb for trout at 1.5° C. is exceptional. Adlung (1957) observed that temperature had little effect on the "absolute lethal dosage" of Thiodan to goldfish.

Table 2:--Toxicity of Thiodan to two species of fish and aquatic invertebrates at two temperatures [Median tolerance limits in parts per billion; 95-percent confidence intervals in parentheses].

Species and temperature	At 24 hours	At 48 hours	At 72 hours	At 96 hours	At 120 hours
Rainbow trout:					
At 1.5° C.	5.9	2.1	1.4	0.8	0.7
	(5.2-6.6)	(1.4-2.8)	---	---	---
At 10° C.	2.1	1.1	0.4	0.3	0.3
	(1.5-2.7)	(0.7-1.5)	---	---	---
Western white sucker:					
At 10° C.	8.1	6.4	4.9	3.5	2.5
	(7.4-8.8)	(5.6-7.2)	---	---	---
At 19° C.	6.6	4.3	3.1	3.0	2.8
	(5.9-7.3)	(3.3-5.3)	---	---	---
Daphnia magna:					
At 10° C.	178.0	132.0	87.5	52.9	47.5
	(162-194)	(113-151)	----	----	----
At 19° C.	68.0	62.0	60.5	56.0	53.5
	(54-82)	(49-75)	----	----	----
Damselfly naiads:					
At 8° C.	235.0	120.0	84.5	71.8	62.0
	(165-305)	(65-180)	----	----	----
At 19° C.	275.0	175.0	150.0	107.0	75.0
	(240-310)	(135-215)	-----	-----	----

The laboratory and field trials with Thiodan reported by other investigators show that it is highly toxic to a variety of species. Adlung (1957) killed goldfish with 10 ppb in 20 hours at 19° to 22° C. Lüdemann and Neumann (1960a) determined a 48-hour LC50 of 11 ppb for carp (Cyprinus carpio) at 17° to 19°. Relative to other organochlorine insecticides, they found that endrin was more toxic by a factor of two and toxaphene less toxic by a factor of five. Also, they later reported that 10 and 5 ppb killed all rainbow trout and northern pike (Esox lucius) respectively within 48 hours at 19° to 21° (Lüdemann and Neumann, 1961a).

F. M. C. Corporation (1958b) applied 46 ppb of Thiodan to a 27-acre pond and killed all minnows, perch, sunfish, bullheads, and suckers within 7 days. Cuerrier (1960) was able to eliminate bluntnose minnows, golden shiners, common suckers, bullheads, perch, smallmouth bass, and sunfish from a lake with a concentration of 15 ppb. In both field trials, there was mortality of frogs and aquatic insects. Plankton declined, but later recovered.

The symptoms of Thiodan poisoning in trout and suckers are similar to those described by other investigators for fish exposed to organochlorine insecticides (Adlung, 1957; Henderson et al., 1959; and Lüdemann and Neumann, 1960a and 1961a). The fish seem overly excitable at first and swim rapidly about the aquariums. Later they surface, lose equilibrium, and move with spasmodic jerks. In time, the majority sink to the bottom, and opercular movements become erratic. Many of the trout become darker, and the suckers appear mottled. Death follows after a variable period.

Some of the trout which survived the 120-hour bioassays at 1.5° C. were placed in fresh aerated tap water. With tactile or vibrational stimuli, many individuals showed symptoms of Thiodan poisoning. Few lived longer than 5 to 7 days after the transfer. Lüdemann and Neumann (1960a) found that the effects of Thiodan on carp, with one exception, were irreversible. One carp exposed to 14 ppb

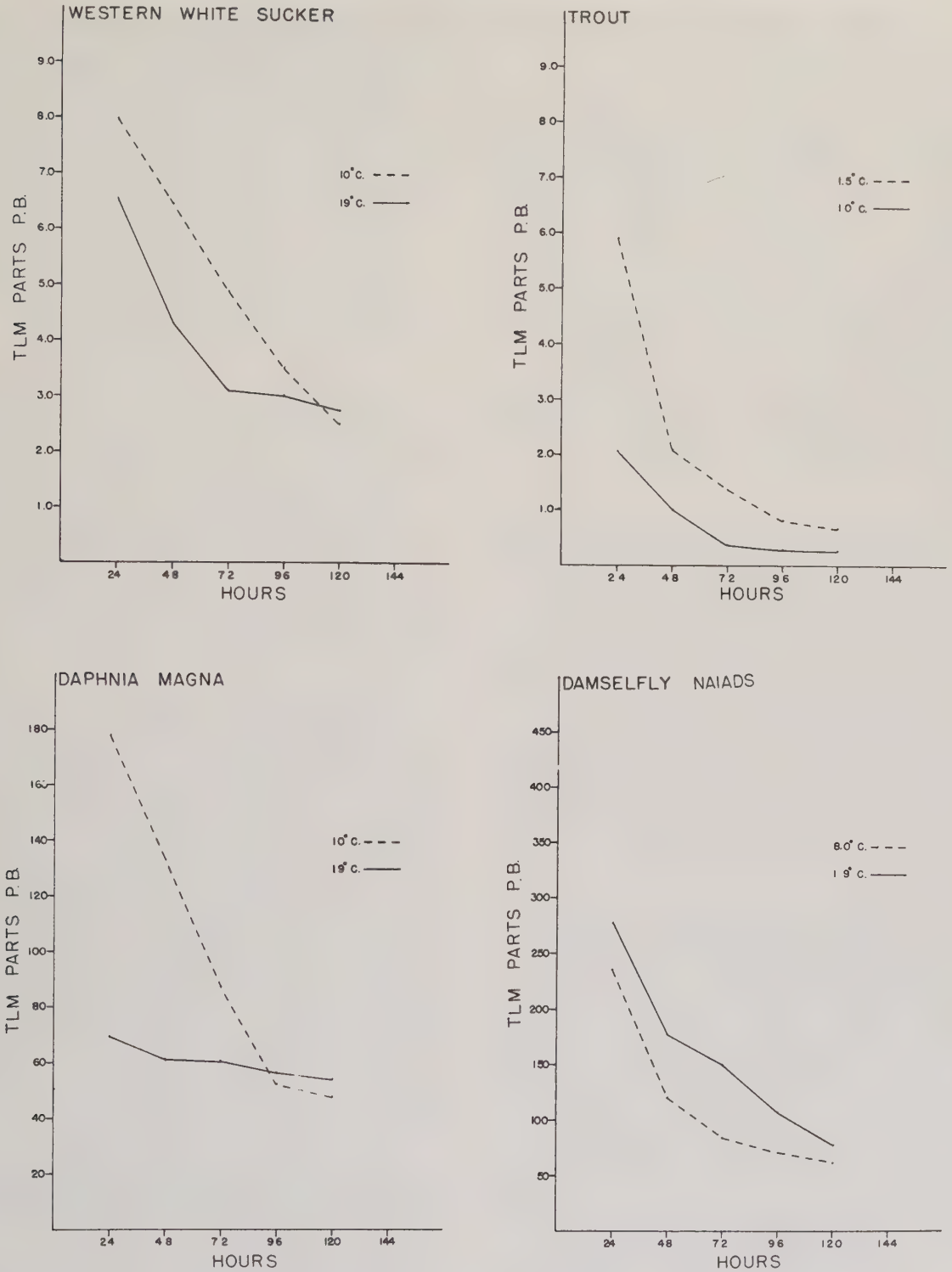

Figure 1:--Effects of time and temperature on the TLm's of rainbow trout, western white sucker, Daphnia magna, and damselfly naiads to Thiodan.

lay on its side after 40 hours, but recovered completely in fresh water.

Thiodan is one of the more toxic chlorinated insecticides to fish, but it is one of the least toxic to mammals. Linquist and Dahm (1957) reported an acute oral LD50 of Thiodan in corn oil to rats of 40 to 50 mg per kg body weight. The manufacturer obtained a value of 110 mg per kg when the insecticide was administered orally in cottonseed oil, and an LD50 of 359 mg per kg when it was applied to the skin of rabbits (F.M.C. Corporation, 1964). Negherbon (1959) cited oral toxicity values in mg per kg for similar chlorinated insecticides dissolved in peanut oil: endrin 16.8; dieldrin 38.3; toxaphene 40.0; and aldrin 45.9.

Loeb (1963) determined the oral toxicity of Thiodan to carp. Technical Thiodan was not toxic in 120 hours at doses of 119 to 234 mg per kg, but doses of 49 and 195 mg per kg of 24-percent miscible Thiodan were lethal within 118 and 22 hours respectively. A dust form of the insecticide was twice as toxic as the miscible formulation. The lethal oral levels for carp are similar to the toxic doses for rats. Although the doses for carp cannot be compared directly with lethal concentrations in water, they suggest that Thiodan is more easily absorbed across gill and oral membranes than by the intestine.

According to my results, and those of others, Thiodan appears to have little value as a selective piscicide against suckers or carp. Concentrations which are lethal to so-called rough or problem species are also toxic to game fish such as trout and northern pike. Fishery biologists recognize, however, that in many instances where fish control is indicated, the proportion of game fish may be insignificant. Here, a nonselective poison such as Thiodan could be used provided it was not overly detrimental to other aquatic organisms and decomposed rapidly into a harmless product.

Fertilized fish eggs--The exposure of rainbow trout eggs to various concentrations of Thiodan for 30 or 120 minutes appears to have no adverse effect on their hatching success (table 3). Development in the eggs had progressed to approximately the 32- or 64-cell stage at the time of treatment. The eggs hatched 29 days after fertilization, and mortalities in the control groups were similar to those observed in groups exposed to concentrations of up to 50,000 ppb. I was unable to test higher concentrations because Thiodan could not be maintained in the test solutions. It formed a noticeable precipitate in the water and adhered to the sides of the containers.

Neither gross nor microscopical observations of treated and control eggs revealed any abnormal development that was attributed to exposure to Thiodan. Large numbers of young fish in the test and control groups died 46 days after hatching. Fish from the same lot of eggs, but used in other experiments at the Fish Control Laboratory, also died. Some factor other than Thiodan was believed responsible for the mortality, although a causative agent could not be established.

Rainbow trout eggs are extremely resistant to Thiodan in comparison with 2-inch fish of the same species. This resistance may be related to selective permeability of the egg chorion or to underdevelopment of Thiodan-sensitive structures at the time of treatment, or both. Antimycin A, a registered fish toxicant which inhibits oxidative-phosphorylation (Walker, et al., 1964), was tested against eggs from the same lot as those in the Thiodan trials. It killed the eggs at a concentration of 10 ppb, which suggests selective permeability of the egg chorion, or at least a mode of action different from that of Thiodan. Exploratory tests with Thiodan and antimycin against the eggs of northern pike support this finding.

Berger[3/] also tested Thiodan against eggs of rainbow trout, but exposed them continuously to the toxicant until they hatched.

3/ Personal communication from Bernard L. Berger, Chemist, Bureau of Sport Fisheries and Wildlife, Fish Control Laboratory, La Crosse, Wis., 1965.

Table 3:--Hatching success of rainbow trout eggs after 30- or 120-minute exposures to various concentrations of Thiodan.

Concentration and exposure	Number of eggs	Number unfer-tilized	Number examined microscopically	Number remaining before hatching	Percent hatching
Control:					
30 minutes	100	1	35	64	76.6
120 minutes	100	7	28	65	70.8
10 ppb:					
30 minutes	100	5	31	64	84.4
120 minutes	100	5	29	66	69.7
100 ppb:					
30 minutes	100	7	31	62	51.6
120 minutes	100	6	29	65	86.2
1,000 ppb:					
30 minutes	100	7	31	62	79.0
120 minutes	100	2	28	70	68.2
10,000 ppb:					
30 minutes	100	6	31	63	76.2
120 minutes	100	0	28	72	94.4
50,000 ppb:1/					
30 minutes	100	3	32	65	73.8
120 minutes	100	3	28	69	85.5

1/ Thiodan formed a white precipitate in these solutions.

The hatched fish were held in the same solutions. He found that levels up to 50,000 ppb were not lethal over the 25-day incubation period, but that the fish became more susceptible after hatching and as they grew and absorbed the yolk sac. Concentrations of 750, 500, and 250 ppb caused 100-percent mortality of trout fry after 7, 12, and 20 days respectively. None of the treated fish survived after 30 days in 100 ppb of Thiodan, although about 30 percent of the controls also died at this time. Iyatomi et al. (1958) observed similar toxicity of endrin to newly hatched carp and snakehead fish (Channa argus).

Daphnia magna--Lüdemann and Neumann (1960b, 1960c, 1961b, and 1962) and Cuerrier (1960) indicate that most aquatic invertebrates, including Daphnia, are less sensitive than fish to Thiodan. My bioassays support this finding (table 2). The 24- to 120-hour TLm's at 19° C. range from 68.0 to 53.5 ppb, and at 10° the values are 178 to 47.5 ppb. The lowest value at 10° indicates that D. magna are at least six times as resistant to Thiodan as suckers or trout. Thiodan used in fish control at concentrations of approximately 50 ppb, as it was in one experimental field trial, could have an adverse effect on Daphnia populations.

Lüdemann and Neumann (1960b) suggest that D. magna are even more resistant to Thiodan than was indicated by my bioassays. They found that 100 ppb caused only partial mortality, and 1,000 ppb killed all of the animals in 24 hours at 18° to 21° C. Extrapolation of the regression for the 24-hour TLm at 19° yields a 100-percent lethal concentration of about 90 to 100 ppb. Since they did not measure the toxicity of concentrations between 100 and 1,000 ppb, levels considerably below the latter value also may be 100-percent lethal.

Thiodan does not appear to have a significant chronic effect on D. magna at 19° C. The TLm values change little with longer exposures (fig. 1). The chemical degradation of Thiodan is a possible explanation. The addition of

sodium bicarbonate to the media containing Daphnia increased pH to about 7.9. In later tests pH 8.4 promoted decomposition of Thiodan, and conceivably pH 7.9 had a similar effect.

At 10° C. Thiodan is initially 2.5 times less toxic to D. magna than at 19° (fig. 1). With longer exposures the toxicity of the compound increases and after 96 hours gives TLm values similar to those at the higher temperatures. The chronic effect of Thiodan at 10° suggests that degradation at alkaline pH is temperature dependent.

Damselfly naiads--Damselfly naiads appear to be the most resistant to Thiodan of the four species of animals tested, except for trout eggs. However, in these trials the chemical was more toxic to naiads in cold water than in warm water. The TLm's at 8° C. ranged from 235 to 62 ppb, whereas at 19° the values were 275 to 75 ppb (table 1). In tests with other species, the compound is usually less toxic at colder temperatures. The greater toxicity of Thiodan to naiads at 8° was unexpected, but a second series of tests supported the earlier results. Since the bioassays extended over a period of 4 to 5 weeks, older naiads were used in the 8° trials. These specimens may have been physiologically more susceptible to Thiodan than younger individuals. McDonald and Jacobson (1958) demonstrated differences in the susceptibilities of army cutworms of various ages to endrin. They found that older larval instars were more resistant. The older damselfly naiads in my trials may have been less resistant. Thiodan appears to have a chronic effect on the insects with time, but levels off at about 60 to 70 ppb after 120 hours of exposure at both temperatures (fig. 1).

According to the results of Stringer and McMynn (1956), and Schoettger and Olive (1961) toxaphene appears relatively more toxic than Thiodan to damselfly naiads. They found that levels of 10 to 30 ppb of the former compound were lethal. However, insects such as Chironomus and Corethra (Chaoborus) are more sensitive to Thiodan than to toxaphene (Lüdemann and Neumann, 1962).

Extrapolations of the 24- and 48-hour regressions indicate that approximately 425 ppb of Thiodan are required to kill 100 percent of the damselfly naiads. This concentration is relatively independent of temperature and exposure.

Effects of water quality on toxicity

Calcium and magnesium salts--Salts of calcium and magnesium in concentrations of 500 ppm, as calcium carbonate hardness, do not alter the toxicity of 20 ppb of Thiodan to suckers (table 4). The mortalities of fish in solutions containing salts and Thiodan, and in those without salts are similar and appear unrelated to aging of the solutions. Statistically, the probability of observing this set of data under the null hypothesis is 0.81. Solutions containing only the salts are not toxic.

pH--Trials with D. magna suggested that alkaline pH may influence the toxicity of Thiodan. Solutions containing 20 ppb of Thiodan were adjusted to pH 6.4 or 8.4 and bioassayed with suckers at 19° C. The results in table 5 indicate that pH has no effect on toxicity. Since the solutions were open to the air and aerated, pH was measured periodically. It declined about 2 to 4 tenths of a pH unit in both acidic and basic solutions during the first few hours of the test. After 24 hours the pH of acidic solutions was approximately 6.2 and that of the basic solutions was 7.4. Although pH control was unsatisfactory, the insecticide did not degrade rapidly in the early segment of the test.

The later determinations of effects of pH on the toxicity of Thiodan were performed by adjusting pH of the solutions and then sealing and storing them for various periods before bioassay. The change in pH was less than 0.75 of a unit over periods of 240 hours using this technique. The chemical degradation of Thiodan with time rather than the immediate effect of pH on toxicity was measured.

The storage of solutions containing 20 ppb of Thiodan at pH 6.4 for periods up to 240 hours has no effect on their toxicity to suckers (table 6). A statistical evaluation of

Table 4:--Effects of calcium and magnesium salt solutions, and solution age, on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Tap water control	Number of fish surviving in-- Tap water containing 500 ppm of $CaCl_2$	$MgCl_2$	$CaSO_4$	$MgSO_4$	20 ppb of Thiodan	20 ppb of Thiodan and 500 ppm of $CaCl_2$	$MgCl_2$	$CaSO_4$	$MgSO_4$
48 hours	10	10	10	10	10	10	0	2	2	0	2
192 hours	10	10	10	10	10	10	3	2	1	2	0
436 hours	10	10	10	10	10	10	1	0	0	1	0

Table 5:--Effects of pH on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Test	Number of fish per jar	Number of fish surviving in-- Tap water control: pH 6.4 replicates 1	2	3	Tap water control: pH 8.4 replicates 1	2	3	20 ppb of Thiodan: pH 6.4 replicates 1	2	3	20 ppb of Thiodan: pH 8.4 replicates 1	2	3
No. 1	10	10	10	10	10	10	10	1	0	0	1	1	1
No. 2	10	10	5	10	10	10	10	0	0	0	1	0	1

Table 6:--Effects of pH 6.4 and 7.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in-- Tap water control: pH 6.4 replicates 1	2	3	Tap water control: pH 7.4 replicates 1	2	3	20 ppb of Thiodan: pH 6.4 replicates 1	2	3	20 ppb of Thiodan: pH 7.4 replicates 1	2	3
120 hours	10	10	10	10	10	10	10	0	0	1	0	0	1
168 hours	10	10	10	10	10	10	10	6	2	7	0	1	0
240 hours	10	10	9	10	10	10	9	0	0	0	1	4	1

mortalities in toxic solutions at pH 6.4 and 7.4 gave a probability under the null hypothesis of 0.04. This is a low probability in comparison with that calculated above for the effect of calcium and magnesium salts. It suggests some degradation of Thiodan in solutions of pH 6.4; however, survival in these vessels did not appear random, but predominated in those aged for 168 hours. The relatively high survival in two jars may be related to an error in the preparation of solutions, or to an inadvertent selection of resistant fish. In general, I considered the experiment adequate and the original conclusion valid.

The aging of alkaline solutions containing 20 ppb of Thiodan reduces or completely eliminates their toxicity to suckers (tables 7 and 8). Solutions of pH 8.4 were only slightly toxic after 72 hours of aging. The mortalities of fish in solutions aged for 24 and 48 hours averaged 60 and 15 percent respectively. Interpolation of these percentages on the 24-hour concentration-mortality regression gives Thiodan concentrations of about 7.3 and 3.8 ppb and indicates a three- to five-fold degradation of the insecticide.

Nearly all of the toxicant degrades within 24 hours at pH 9.4 (table 8). Mortalities were

Table 7:--Effects of pH 7.4 and 8.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in-- Tap water control: pH 7.4	Tap water control: pH 8.4	20 ppb of Thiodan: pH 7.4	20 ppb of Thiodan: pH 8.4 replicates 1	2	3	4
24 hours:								
Test No. 1	10	10	10	1	0	10	0	3
Test No. 2	10	10	10	1	8	3	7	2
48 hours:								
Test No. 3	10	10	10	2	10	10	10	6
Test No. 4	10	10	10	0	8	10	9	6
72 hours:								
Test No. 5	10	10	10	1	10	10	10	10
Test No. 6	10	10	10	1	7	10	10	10
96 hours:								
Test No. 7	10	10	10	2	10	8	10	10
Test No. 8	10	10	10	1	10	10	10	10

Table 8:--Effects of pH 7.4 and 9.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in-- Tap water control: pH 7.4	Tap water control: pH 9.4	20 ppb of Thiodan: pH 7.4	20 ppb of Thiodan: pH 9.4 replicates 1	2	3	4
24 hours:								
Test No. 1	10	10	10	5	10	10	10	10
Test No. 2	10	10	10	2	8	10	10	10
48 hours:								
Test No. 3	10	10	10	3	10	10	8	9
Test No. 4	10	10	10	0	10	10	10	10
72 hours:								
Test No. 5	10	10	10	3	10	10	10	10
Test No. 6	10	10	10	0	10	10	10	8
96 hours:								
Test No. 7	10	10	10	1	10	10	10	10
Test No. 8	10	10	10	0	10	10	10	10

observed in only 4 of 32 potentially toxic solutions and none exceeded 20 percent. All of the control fish survived at pH 7.4 to 9.4 and the mortalities in 20 ppb of Thiodan at pH 7.4 ranged from 50 to 100 percent.

Statistical probabilities for observing certain blocks of data in tables 7 and 8 were computed under the null hypothesis (table 9). The relative magnitude of the probabilities was considered in assigning significance to the various comparisons. The very low probability that the survival of suckers in Thiodan solutions aged for 24 hours at pH 8.4 was no different from that of fish in the control (comparison 2) indicates residual toxicity. The higher probability

Table 9:--Probabilities, under the null hypothesis, of observing various sets of data taken from experiments on pH.

Comparison	Table number	Age of solutions (hours)	Comparison	Probability
No. 1	7	24	Survival in 20 ppb of Thiodan, pH 7.4, vs. Survival in 20 ppb of Thiodan, pH 8.4	0.0064.
No. 2	7	24	Survival in control, pH 8.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	Approximately 3.78^{-47}.
No. 3	7	48, 72, 96	Survival in 20 ppb of Thiodan, pH 7.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	Approximately 1.03^{-36}.
No. 4	7	48, 72, 96	Survival in control, pH 8.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	0.0254.
No. 5	8	24, 48, 72, 96	Survival in 20 ppb of Thiodan, pH 7.4 vs. Survival in 20 ppb of Thiodan, pH 9.4	Approximately 5.33^{-53}.
No. 6	8	24, 48, 72, 96	Survival in control, pH 9.4 vs. Survival in 20 ppb of Thiodan, pH 9.4	0.2069.

for comparison 1, however, suggests that some detoxification of the chemical occurs with 24 hours. The aging of similar solutions for longer periods (comparisons 3 and 4) results in further detoxification which is demonstrated by a reversal in magnitude of probabilities for the respective comparisons. The values for comparisons 5 and 6 indicate extensive detoxification of Thiodan at pH 9.4.

Of the various water-quality factors which were considered as possibly important in the detoxification of Thiodan, alkaline pH is the most significant. Calcium and magnesium ions and mildly acid media appear to have no effect on toxicity. Thus, the residual toxicity of Thiodan can be minimized when treatments are made just prior to anticipated increases in basicity. We must not discount the interaction of temperature and pH on the residual toxicity of Thiodan. The efficacy of alkaline pH on detoxification of Thiodan at temperatures lower than 19° C. may be considerably less. Berger[4/] found that 9 days were required to detoxify 10 ppb of Thiodan to rainbow trout at pH 9 and 12° C.

According to Frensch et al. (1959) the fate of Thiodan in water is hydrolysis to Thiodan alcohol. Apparently little is known of the biological toxicity of the alcohol, although it

4/ Personal communication from Bernard L. Berger, Chemist, Bureau of Sport Fisheries and Wildlife, Fish Control Laboratory, La Crosse, Wis.

must be relatively nontoxic to fish. Lindquist and Dahm (1957) tested it against one rat, and an oral dose of 1370 mg per kg was not lethal.

Deposition and metabolism of Thiodan

Radioactive Thiodan--The exposure of suckers to 20 ppb of ^{14}C-labeled Thiodan results in the deposition of significant amounts of radioactive substances in their tissues (tables 10, 11, and 12). The mean concentrations of labeled substances in µg per g of dry tissue during 12 hours of exposure are: liver 8.4; gut and feces 5.8; blood 4.4; heart 4.1; gill 3.1; kidney 2.7; gut (empty) 2.7; brain 2.6; skin 1.8; and muscle 1.1.

A concentration of 80 ppb of ^{14}C-labeled Thiodan killed suckers within 2.25 and 9.25 hours of exposure. The mean levels of radioactive compound in their tissues in µg per g of dry tissue are: liver 22.9; gut and feces 14.5; blood 8.9; brain 6.5; kidney 6.0; heart 5.6; gut (empty) 5.3; gill 4.0; skin 2.6; and muscle 1.8 (tables 10, 11, and 12). The livers, guts, blood, brains, and kidneys of fish treated with 80 ppb of ^{14}C-labeled Thiodan contain approximately 2 to 3 times as much radioactive material as was present in the same tissues of fish treated with, but not killed by, a concentration of 20 ppb. The activity levels in several tissues of the former seem highly variable and poorly correlated with exposure. Those in brain, on the other hand, were relatively consistent and may represent the concentration necessary to cause death.

The concentrations of radioactive substances, presumably ^{14}C-labeled Thiodan, in muscles of suckers are relatively low in comparison with those in other tissues. This tissue, however, is probably one of the larger reservoirs for Thiodan deposition because of its relatively greater mass. In order to estimate the total Thiodan deposited in muscle in relation to that added to the external medium, the percent of muscle in the fish and its water content must be considered. Holden (1962) assumed that muscle amounted to 75 percent of the total body weight of brown trout, and Spector (1956) placed the water concentrations in muscles of several freshwater fish at about 70 percent. Using these percentages, an average weight of fish of 40 grams, and average levels of Thiodan in muscle, I calculated that 9.9 µg of Thiodan were present in the muscle of each fish treated at 20 ppb and 16.2 µg in those killed by 80 ppb. The deposits in muscle comprise 33 and 14 percent respectively of the total ^{14}C-labeled Thiodan added to the external media. A fourfold increase of the Thiodan concentration in the water did not elevate muscle residues by a similar amount.

The small difference between muscle residues of fish treated at 20 or 80 ppb of ^{14}C-labeled Thiodan suggests that the insecticide may reach a level of saturation in muscle lipids. Ludwig, Weis, and Korte (1964) found that aldrin reached a level of saturation in the lipids of rats after which no additional toxicant was stored.

Linear regressions based on the unaveraged tissue concentrations of radioactive substances and exposures were calculated for fish treated at 20 ppb of ^{14}C-labeled Thiodan to estimate correlation and rate of uptake (fig. 2 and table 13). The correlation coefficients for liver, gut and feces, blood, heart, gill, kidney, and brain range from 0.67 to 0.97 and are statistically significant at the 0.05 level of probability. The concentrations of labeled substances in gut (empty), skin, and muscle are not significantly correlated with exposure, particularly those in muscle, which had a coefficient of only 0.29. The tissues containing the greatest concentrations of radioactivity are not necessarily the ones with the highest rates of uptake. For example, gut and feces contain a lower average concentration than liver, but the rate of uptake in the former was approximately 22 percent greater. The intercepts and slopes of the regressions suggest that the uptake of ^{14}C-labeled Thiodan by the various tissues is rapid and probably nonlinear during the first hour of exposure. Thus, the linear regressions cannot be extrapolated linearly to determine uptake rates for the first hour.

Table 10:--Concentrations of radioactive substances in liver, kidney, and gut of western white suckers following various exposures to two concentrations of ^{14}C-labeled Thiodan at 19° C.

Concentration of ^{14}C-labeled Thiodan	Hours Exposure	Number of fish	Radioactive substances (µg/g dry weight of tissue) in Liver Mean	Liver Counting error[1] (±)	Kidney Mean	Kidney Counting error[1] (±)	Gut Mean	Gut Counting error[1] (±)
20 ppb	1.00	2	4.3	0.4	1.5	0.6	1.2	0.4
20 ppb	2.00	1	4.2	0.4	2.2	0.9	1.8[3]	0.9
20 ppb	3.00	2	6.3	0.5	2.6	0.5	3.7	0.5
20 ppb	4.00	1	5.0	0.5	1.3	0.5	2.3[3]	1.1
20 ppb	6.00	2	12.2[2]	0.4	3.9	0.7	7.2	0.5
20 ppb	8.00	1	10.4	0.8	4.0	1.2	3.8[3]	1.2
20 ppb	9.00	2	13.9	0.5	2.3	0.4	10.9	0.7
20 ppb	12.00	1	11.1	0.9	4.4	1.2	3.0[3]	1.1
Overall mean			8.4	0.6	2.7	0.8	5.8	0.3
							2.7[3]	1.1
80 ppb	2.75[4]	1	18.8	0.5	4.0	0.6	6.9	0.1
80 ppb	5.50[4]	2	35.0[2]	0.9	9.5	0.6	22.1	1.0
80 ppb	9.25[4]	1	14.8	0.7	4.5	0.5	5.3[3]	0.8
Overall mean			22.9	0.7	6.0	0.6	14.5	0.6
							5.3[3]	0.8

1/ 0.05 probability limit.
2/ One sample.
3/ Gut contents removed.
4/ Time of death.

Table 11:--Concentrations of radioactive substances in blood, heart, and brain of western white suckers following various exposures to two concentrations of ^{14}C-labeled Thiodan at 19° C.

Concentration of ^{14}C-labeled Thiodan	Hours Exposure	Number of fish	Radioactive substances (µg/g dry weight of tissue) in Heart Mean	Heart Counting error[1] (±)	Brain Mean	Brain Counting error[1] (±)
20 ppb	1.00	2	2.1[2]	0.2	2.2	0.7	1.5	0.4
20 ppb	2.00	1	3.5	0.9	4.5	1.6	2.3	0.7
20 ppb	3.00	2	---	---	2.6	0.8	1.8	0.4
20 ppb	4.00	1	3.9	0.7	2.5	1.7	1.6	0.6
20 ppb	6.00	2	---	---	4.2	0.9	3.5	0.4
20 ppb	8.00	1	5.3	1.7	5.3	1.7	2.8	0.7
20 ppb	9.00	2	---	---	4.0	0.7	3.4	0.4
20 ppb	12.00	1	7.0	0.9	9.7	4.0	3.7	0.1
Overall mean			4.0	0.9	4.1	1.5	2.6	0.6
80 ppb	2.25[3]	1	---	---	5.9	0.6	6.2	0.4
80 ppb	5.50[3]	2	11.1	0.1	7.3	1.1	7.3	0.6
80 ppb	9.25[3]	1	6.7	0.4	3.7	1.2	5.0	0.7
Overall mean			8.9	0.3	5.6	1.0	6.5	0.6

1/ 0.05 probability limit. 2/ One sample. 3/ Time of death.

Table 12:--Concentrations of radioactive substances in skin, gill and muscle of western white suckers following various exposures to two concentrations of ^{14}C-labeled Thiodan at 19° C.

Concentration of ^{14}C-labeled Thiodan	Hours Exposure	Number of fish	Radioactive substances (µg/g dry weight of tissue) in Skin Mean	Skin Counting error 1/ (±)	Gill Mean	Gill Counting error 1/ (±)	Muscle Mean	Muscle Counting error 1/ (±)
20 ppb	1.00	2	1.1	0.8	1.9	0.5	1.2	0.3
20 ppb	2.00	1	0.6	0.7	2.7	1.6	0.5	0.2
20 ppb	3.00	2	2.5	1.1	3.7	1.7	0.8	0.2
20 ppb	4.00	1	1.1	1.1	3.1	1.5	0.6	0.2
20 ppb	6.00	2	2.4	0.9	2.8	1.1	1.8	0.3
20 ppb	8.00	1	1.2	0.5	2.4	0.5	1.3	0.4
20 ppb	9.00	2	1.3	0.8	3.2	0.9	1.1	0.3
20 ppb	12.00	1	4.9	2.9	6.4	1.7	1.3	0.4
Overall mean			1.8	1.1	3.1	1.2	1.1	0.3
80 ppb	2.25 2/	1	3.1	0.9	4.1	0.4	1.4	0.2
80 ppb	5.50 2/	2	3.3	0.4	4.9	0.7	3.3	0.3
80 ppb	9.25 2/	1	1.3	1.1	3.1	0.5	0.8	0.2
Overall mean			2.6	0.8	4.0	0.5	1.8	0.2

1/ 0.05 probability limit.
2/ Time of death.

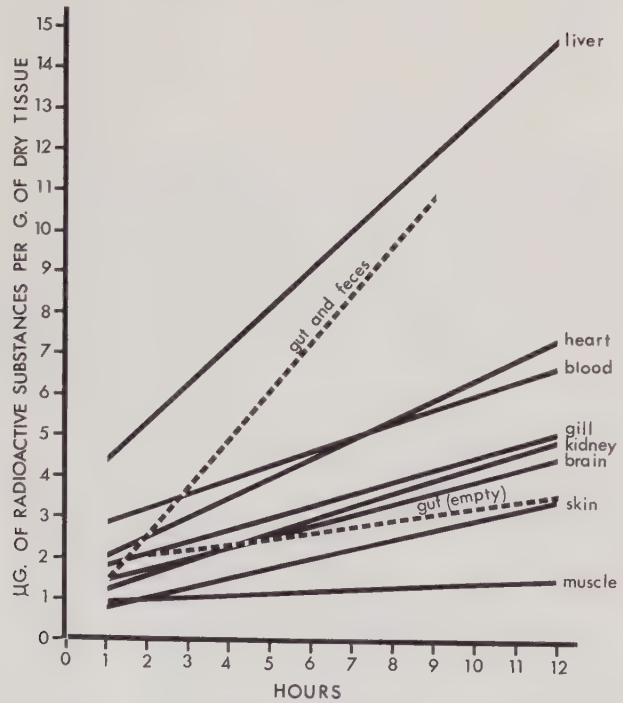

Figure 2:--Uptake of radioactive substances in tissues of western white suckers during their exposure to 20 ppb of ^{14}C-labeled Thiodan at 19° C.

Table 13:--Slopes and correlation coefficients for regressions in figure 2.

Tissue	Slope (b)	Correlation coefficient (r)
Liver	0.93	0.85
Gut and feces	1.18	0.97
Heart	0.48	0.78
Blood	0.34	0.89
Gill	0.29	0.67
Kidney	0.33	0.84
Brain	0.25	0.82
Gut (empty)	0.14	0.66
Skin	0.23	0.48
Muscle	0.05	0.29

In general, with the exception of gut and feces, the higher concentrations and rates of uptake of labeled chemical occur in blood and those tissues containing relatively large amounts of blood. The actual volumes of blood in sucker tissues were not measured; however, Hoffert (1966) found that livers, gills, kidneys, and muscles of lake trout contained 17.0, 16.5, 8.9, and 0.4 percent blood respectively. If

similar amounts of blood are present in sucker tissues, and if the concentrations of radioactive substances were wholly dependent on blood content in tissue, then one would expect to detect concentrations of radioactivity in tissues which vary according to blood content, and the radioactivity in blood. The regressions in figure 2 indicate that radioactive substances in liver, gill, kidney, and muscle were not sufficiently lower than those in blood to be correlated entirely with the blood content of the tissues. Since the ranking for concentrations of radioactivity in gill, kidney, and muscle was similar to Hoffert's blood values, the blood content may have some influence on the level of radioacitivity in the tissue.

The observation that the levels of labeled substances in the liver were much greater than those in blood suggests that Thiodan is removed from the blood by this organ and stored. The lower deposition and uptake in other tissues, except gut and feces, may be due to this function of the liver.

The removal of the contents of the gut lowers the concentration of radioactivity in the gut (table 10 and fig. 2). This demonstrates that the majority of the radioactivity present in the intestine is contained in the feces rather than in the tissue itself. Mount (1962) treated bluntnose minnows with endrin and detected it within the gut. In his opinion, endrin may have entered the tract along with ingested water. Because few fresh water teleosts drink significant amounts of water (Black, 1957), another avenue for the entrance of Thiodan into the intestinal tracts of suckers was sought. Since the samples of gut used in the analyses were collected posterior to the entrance of the gallbladder, I postulated that the radioactivity may have entered with the bile.

A second series of experiments was necessary to test the hypothesis that Thiodan is excreted in bile. This also provided an opportunity to evaluate the solubility characteristics of the labeled materials in tissues and bile of ^{14}C-labeled Thiodan treated fish. Northern creek chubs and western white suckers were exposed to 40 ppb of ^{14}C-labeled Thiodan for 3 or 5 hours at 21° C. The concentrations and relative benzene-water solubilities of radioactive substances in the bile, and in chub tissues are shown in tables 14 and 15. Simple, ethanolic extracts of chub tissues removed approximately 62 to 100 percent of the radioactivity (table 14). The majority of the labeled substances in the lipid extracts are separated, after benzene-water partition, into the benzene fraction. The remainder, ranging from 9 to 20 percent of the total, appear water soluble; however, a portion or all of these may have been benzene soluble since emulsions were included with this fraction.

The concentrations of benzene-soluble radioactive substances are highest in liver, brain, kidney, and blood, in that order (table 14). Extension of the exposure from 3 to 5 hours did not increase the amounts deposited in the tissues.

Conclusions about distribution of Thiodan based on measurements of radioactivity must be qualified because of the possible metabolic destruction of the chemical. The fact that the radioactive substances extracted from chub tissues were soluble in ethanol and benzene indicates that activity levels in chubs and those measured earlier in suckers, represent deposits of Thiodan, or at least the aromatic portion of the molecule. According to Hawk, Oser, and Summerson (1954), aromatic compounds are fairly resistant to oxidation, whereas their acyclic side chains may be catabolized.

Radioactive materials are present in the bile of chubs and suckers which are treated with ^{14}C-labeled Thiodan (table 15). Ninety-four to 100 percent of the activity is separated, by benzene-water partition, into the water fraction. The concentrations in this fraction range from 88 to 158 μg per g of dried bile for chub and 36.5 to 48.0 μg per g for sucker. Semiqualitative measurements were made of the water-soluble metabolite in sucker bile. Incubation of the bile with beta-glucuronidase converted the labeled metabolite to a benzene-

Table 14:--Benzene- and water-soluble radioactive substances in extracts of tissues of northern creek chubs exposed to 40 ppb of ^{14}C-labeled Thiodan at 21° C.

Tissue and exposure	Number of fish	Radioactive substances (µg/g dry wt. of tissue) in: Benzene soluble fraction Mean	Benzene soluble fraction Counting error 1/ (±)	Water soluble fraction Mean	Water soluble fraction Counting error 1/ (±)	Extracted tissue Mean	Extracted tissue Counting error 1/ (±)	Percent in benzene	Percent in water	Percent in tissue
Liver:										
3 hours	2	8.2	0.4	1.0	0.2	1.7	0.4	75.0	9.0	15.6
5 hours	2	4.5	0.2	0.7	0.2	1.8	0.3	64.0	10.0	25.7
Kidney:										
3 hours	2	3.3	0.5	0.9	0.4	0.3	0.3	73.3	20.0	6.7
5 hours	2	4.2	0.8	0	-	2.6	0.6	61.8	0	38.2
Blood:										
3 hours	2	2.3	0.3	0.6	0.3	0.4	0.2	69.7	18.2	12.1
5 hours	1	2.0	0.5	0.4	0.3	0	-	83.3	16.7	0
Brain:										
3 hours	2	5.3	0.6	0	-	0	-	100	0	0
5 hours	2	3.1	0.6	0	-	0	-	100	0	0

1/ 0.05 probability limit

Table 15:--Benzene- and water-soluble radioactive substances in gallbladder bile of two species of fish exposed to 40 ppb of ^{14}C-labeled Thiodan, and the effect of beta-glucuronidase on substance solubility.

Species and fish number	Exposure	Before incubation of bile with enzyme; radioactive substances (µg/g of dry bile) in--- Benzene soluble fraction: Concentration	Counting error 1/ (±)	Percent	Water soluble fraction: Concentration	Counting error 1/ (±)	Percent	After incubation of bile with enzyme; radioactive substances (µg/g of dry bile) in--- Benzene soluble fraction: Concentration	Counting error 1/ (±)	Percent	Water soluble fraction: Concentration	Counting error 1/ (±)	Percent
Northern creek chub:													
No. 1	3	5.6	0.9	6.0	88.0	5.5	94.0	-	-	-	-	-	-
No. 2	3	0.0	-	0.0	158.0	5.8	100.0	-	-	-	-	-	-
No. 3	5	4.5	0.9	3.5	124.0	5.9	96.5	-	-	-	-	-	-
Western white sucker:													
No. 4	5	1.5	0.5	3.9	36.5	1.7	96.1	46.0	1.7	99.4	0.3	0.6	0.6
No. 5	5	2.2	0.5	4.4	48.0	1.7	95.6	41.4	1.0	97.2	0.1	0.6	2.8

1/ 0.05 probability limit.

soluble form. Thus, the results suggest that the liver removes Thiodan from the blood stream, changes it to an aromatic metabolite, and conjugates it with glucuronic acid. Then the conjugate is discharged with bile into the gallbladder. The subsequent release of bile containing the radioactive conjugate into the gut may explain the high levels of radioactivity present in feces of suckers (fig. 2).

Metabolism and excretion of Thiodan in fish appears to be similar to that observed in rats and rabbits for the excretion of other chlorinated insecticides, such as ^{14}C-labeled aldrin and ^{14}C-labeled dieldrin. These compounds are metabolized by the liver into more hydrophilic compounds and excreted via the bile into the intestinal canal (Korte, Ludwig, and Vogel, 1962; and Morsdorf et al., 1963).

Ludwig et al. (1964) found that rats fed low doses of ^{14}C-labeled aldrin over long periods excreted aldrin, dieldrin, and unidentified hydrophilic products in both feces and urine. The total activity excreted daily over 12 weeks increased from approximately 50 to 100 percent of the daily dose. The amount excreted in the feces was 10 to 20 times as great as that in urine. The hydrophilic compounds in feces and urine were not alike chromatographically, and alkaline hydrolysis of the urinary metabolites gave a compound with acidic properties. Aromatic acids such as benzoic acid are conjugated with glucuronic acid (Long, 1961).

Additional evidence supporting the proposed pathway for the metabolism and excretion of Thiodan by fish will be discussed in the next section.

Nonradioactive Thiodan--As stated earlier, further trials with radioactive Thiodan were impossible owing to its degradation to what appeared to be Thiodan alcohol. To strengthen the findings of the radiation experiments, additional fish were treated with technical Thiodan, and its residues or metabolites in tissues and bile were analyzed chemically.

In preliminary experiments, goldfish and carp were killed with 20 ppb of Thiodan and their tissues analyzed. Traces of the insecticide were found in muscle, but the levels were beyond the range of accurate measurement by the analytical method. The possibility that concentrations of Thiodan in the tissues could be elevated by exposing the fish for longer periods to lower concentrations was tested by treating one goldfish and one carp daily with 7.0 ppb. The carp died after the second treatment and the goldfish after ten. Only traces of the toxicant were found in carp, but 1.1 µg per g wet weight were measured in muscle of the goldfish and 0.5 µg per g in its gills. Seven µg per ml were found in the bile after incubation with beta-glucuronidase. Since only traces of Thiodan were found in other goldfish tissues, samples from a number of fish were combined in later trials in order to measure Thiodan in these tissues satisfactorily. Repeated treatments of goldfish with ordinarily subacute concentrations apparently cause mortality and induce measurable residues of Thiodan.

Ten goldfish weighing 96 to 337 grams were exposed daily and individually to a concentration of 7.0 ppb of Thiodan. The order in which the fish died according to their weight, sex, length of exposure, and total Thiodan treatment is shown in table 16. The two smallest specimens were males, and they succumbed on the fourth and fifth days of treatment after exposure to combined concentrations of 28 and 35 ppb of Thiodan respectively. The remaining fish were females which died at intervals up to 13 days after the initial exposure. Two of the largest individuals survived 20 applications, or exposure to a total of 2.10 mg of Thiodan.

The largest deposits of Thiodan residues in goldfish tissues ranged from 3.40 to 12.76 µg per g of wet tissue in the bile, liver, brain, and abdominal fat respectively (table 16). The mean residues in µg per g of wet tissue for other tissues are: scales and fins 0.45; gonad 0.67; gill 0.86; heart, kidney, spleen, and blood 1.39; muscle 1.67; and skin 2.48. Thiodan was not detected in the gut and feces.

Susceptibility of goldfish to repeated subacute concentrations of Thiodan is strongly related to their weight. The lightest individuals are the most susceptible. The correlation of weight of fish with number of treatments before their death gives a coefficient of 0.89.

The correlation of weight with number of treatments suggests an accumulation of

Table 16:--Residues of Thiodan in goldfish treated daily in fresh solutions containing 7 ppb of Thiodan at 12° C.

Fish	Weight	Sex	Thiodan treatments: Number before death	Thiodan treatments: Amount before death (mg)(ppb)	Concentration of Thiodan in µg per g wet weight in---: Muscle	Bile[1]	Gill	Skin	Scales fins	Liver	Gut and content	Gonad	Heart kidney spleen blood	Brain[2]	Peritoneal fat[3]
No. 1	109	M	4	0.42 28	1.41										---
						0	Samples composed of tissues of fish 1 to 5								
No. 2	96	M	5	0.53 35	2.54		0.69	3.28	0.77	5.61	0	0.73	1.23		---
No. 3	129	F	7	0.74 49	2.10										
						0									---
No. 4	142	F	8	0.84 56	1.15										
No. 5	188	F	11	1.16 77	----										
						3.67									
No. 6	191	F	13	1.37 91	1.99		Samples composed of tissues of fish 6 to 10								
														8.55	---
No. 7	184	F	13	1.37 91	2.13										---
						3.40									
No. 8	277	F	13	1.37 91	0.95		1.03	1.69	0.21	5.32	0	0.61	1.54		---
No. 9	241[4]	F	20	2.10 140	1.09	----									12.76
No.10	338[4]	F	20	2.10 140	----										
Mean					1.67	3.53	0.86	2.48	0.45	5.47	0	0.67	1.39	8.55	12.76

1/ Samples composed of bile of two fish. Bile incubated with beta-glucuronidase. Mean based on positive values only.
2/ Sample composed of brains of 10 fish.
3/ Sample composed of fat from fish 9 and 10.
4/ Alive after 20 treatments.

Thiodan with time; larger fish, because of greater mass, require a longer exposure to attain the same concentration of Thiodan in their tissues as smaller fish. Residues in fish treated for up to 20 days are essentially no greater than those in individuals receiving up to 11 treatments. The coefficient of correlation for residues in muscle, based on wet weight, with days of exposure is 0.38.

The differences in sizes of the goldfish are due primarily to weight rather than length. The heaviest fish weighed approximately three times as much as the smallest but was only 30 percent longer. This length-weight relation suggests that the heavier and more resistant individuals also contained the greatest amount of lipids. The lipids in goldfish muscle were calculated as a percent of the wet weight of the tissue and for fish 1 to 10 (table 16), are: 1.2, 3.5, 2.1, 3.4, 6.7, 3.0, 5.6, 3.7, 6.8, and 6.1 percent. In terms of dry weight, most of the values are between 14 and 26 percent. The correlation of lipid content with the number of treatments which they survived gives a coefficient of 0.77 indicating that individuals with more muscle lipids survive longest.

The male goldfish were the most susceptible to Thiodan, but also they were the smallest specimens. These two variables were confounded, and the independent influence of sex could not be evaluated.

The micrograms of Thiodan per gram of muscle lipids extracted from each of the 10 fish are, in order: 113.28, 71.93, 97.73, 33.68, 64.37, 37.92, 25.39, and 16.15. The concentrations are negatively correlated with the number of treatments, weight of fish, and the lipid content of the muscle. The coefficients are 0.78, 0.75, and 0.85 respectively. Whereas the quantity of the Thiodan in muscle was relatively constant, an increase in lipid content

allows a decline of Thiodan in muscle lipids of heavier fish. These data indicate that muscle lipids aid in the "detoxication" of Thiodan by providing a reservoir for its storage and dilution. However, since all but the two most resistant goldfish died during the experiment the actual amount of poison in muscle appears to be more closely allied with mortality than its concentration in lipids. Thus, a physically greater mass of lipids in heavier fish contribute more to their survival than the degree of saturation of lipids with Thiodan. The high concentration of Thiodan in abdominal fat of fish 9 and 10 (table 16) indicates that insecticide storage in this tissue may also contribute to the resistance of the heavier individuals.

Thiodan concentrations are considerably greater in brain than those in gill, liver, and a composite of heart, kidney, spleen, and blood (table 16). This is contrary to observations made on suckers where the high concentrations of what was presumed to be Thiodan were associated with blood containing tissues. Also, the skin of suckers contained less Thiodan than that of goldfish. This apparent inconsistency may be related to a greater lipid content of goldfish, and their longer exposure to Thiodan.

Thiodan is adsorbed on or absorbed into the scales and fins of goldfish (table 16). Although the levels are comparatively low, they demonstrate an ability of the body surfaces to remove and concentrate the insecticide from the external medium. Holden (1962) found that DDT was concentrated by the external mucus of brown trout, and he pointed out the possibility of its entry into fish through the skin.

The gonads of suckers treated with ^{14}C-labeled Thiodan were not assayed for radioactivity, but composite samples of goldfish gonads contain 0.67 to 0.73 µg of Thiodan per g of wet tissue (table 16). In toxicity trials, the fertilized eggs of rainbow trout were extremely resistant to Thiodan; however, incorporation of the insecticide into fish eggs before spawning may have an adverse effect on their fertility or survival. Residues of chlorinated insecticides such as DDT in fish eggs are suspected of reducing their reproductive success (Burdick et al., 1964; Cuerrier, Keith, and Stone, 1967; and Macek, 1968).

The water content of samples of goldfish muscle ranged from 71.3 to 78.1 percent and averaged 75.2 percent. The two fish receiving 20 treatments with Thiodan (table 16) had the lowest water concentrations in their muscles. In general, however, there appeared to be no relation between the mortality of fish and tissue water.

The measurement of Thiodan in various tissues of goldfish by relatively specific chemical methods supports an earlier belief that the majority of the radioactive substances detected in suckers were indeed ^{14}C-labeled Thiodan. According to Barnes and Ware (1965) technical Thiodan contains four components, the high and low melting point isomers, Thiodan alcohol, and Thiodan ether. The chemical method used in my investigation measures both isomers and the Thiodan alcohol but not the ether. The low melting point isomer and the alcohol are not recoverable from the Florisil-carbon column. The possibility that a small portion of the radioactivity in suckers did not represent Thiodan is suggested by the incomplete extractions of radioactive substances from some chub tissues with ethanol (table 14). The remaining activity may possibly have been associated with water-soluble metabolites.

Thiodan is not detected colorimetrically in petroleum ether extracts of goldfish bile until after the samples are incubated with beta-glucuronidase, nor in extracts of gut and content (table 16). This was expected considering the existence of water-soluble radioactive substances found in the bile of suckers and chubs (table 15). The wavelength of maximum absorption for the colored product is 534 mµ, the exact wavelength used for measurements of Thiodan. Its average concentration in bile is 3.5 ug per ml, after applying a correction for a background of unidentified constituents of the bile. The uncorrected concentrations agree closely with that measured in bile during the preliminary investigation. The average

background was 3.6 µg per ml. It was derived by measuring the absorbance, at 534 mµ, of two samples which apparently contained no Thiodan; one made up of bile from fish 1 and 2 and another from fish 3 and 4. In these samples, peaks of maximum light absorption were not detected at 534 mµ.

The apparent lack of Thiodan metabolites in the bile of some goldfish may be attributed to: amounts below the limits of detectability due to small volumes of bile or slow rates of conjugate formation; or inhibition of beta-glucuronidase. The volumes of bile in fish 1 to 4 were 0.4 to 0.5 ml whereas those in the remaining fish usually exceeded 0.7 ml. Ludwig et al. (1964) found that rats excreted greater amounts of aldrin metabolites the longer they were maintained on an aldrin containing diet. Beta-glucuronidase is inhibited by several substances including a product of the hydrolysis, glucuronic acid (Long, 1961).

The hydrolysis of water-soluble metabolites of Thiodan, in experiments with both suckers and goldfish, with beta-glucuronidase indicates that these species are able to metabolize the insecticide and conjugate it with glucuronic acid. According to Harper (1959), in mammals uridine diphosphate glucuronic acid (UDPGA) is the active form of the acid involved in the conjugation of chemicals containing carboxyl and hydroxyl groups. It is formed by the oxidation of UDP-glucose which is catalyzed by a diphosphopyridine nucleotide-dependent UDPG dehydrogenase. The conjugation reaction requires glucuronyl transferase. My results imply that not only are both enzymes present in the livers of these species, but also that this organ is able to convert the insecticide to a substance containing carboxyl or hydroxyl groups. The conjugable substance is probably Thiodan alcohol since it, like Thiodan, reacts colorimetrically.

Barnes and Ware (1965) studied the metabolism of Thiodan in house flies. It was oxidized to Thiodan sulfate, a slightly less toxic compound which was considered to be an intermediate in the detoxication process. Their analyses of feces revealed water- and acetone-soluble substances which they believed were glucoside, or glucosiduronic conjugates of Thiodan metabolites. Although they did not establish the identity of the conjugate, they did eliminate the possibility of conjugation at the terminal sulfur of Thiodan. Thus, experiments with fish and house flies suggest at least one pathway for the biochemical degradation of Thiodan. First, oxidation to Thiodan sulfate, then conversion to Thiodan alcohol, followed by conjugation with glucuronic acid and excretion in the feces. The results of Barnes and Ware (1965) also indicate that the resistance of flies to Thiodan may be controlled by the rate of its oxidation, or the rate of conjugation of the metabolite.

The lipids of fish may serve as a reservoir for depositing the quantities of Thiodan which exceed those that can be immediately and effectively detoxified in the degradation pathway. Then, the deposits may be released and detoxified gradually during the turnover of lipids. Ludwig et al. (1964) reported that rats which were given daily sublethal doses of aldrin in their food were eventually able to excrete the total daily dose as hydrophilic metabolites. After the last dose, the rate of metabolite excretion declined slowly as did the concentration of aldrin in the fat.

In the trials with goldfish, Thiodan may have been released from muscle lipids and its toxicity added to that of later treatments because the fish were fasted throughout the tests. The replenishment of muscle and liver glycogen in fish is dependent on food or conversion from protein and fat (Black et al., 1960; Change and Idler, 1960), and it is conceivable that Thiodan was liberated during fat catabolism. Graham (1960) believed that poor condition and physiological stress of fish may result in stores of DDT becoming lethal.

Another nutritional aspect to be considered in the toxicity of Thiodan to fish is the systemic level of precursors available for the formation of glucuronic acid. As mentioned earlier, glucose is the forerunner of glucuronic

acid in the uronic acid pathway. Therefore, deficiencies in glucose or glycogen might retard the conjugation of Thiodan metabolites because of insufficient glucuronic acid. Brodie and Maickel (1962) found that frogs which were pre-fed glucose converted phenols largely to glucosiduronic acids. Without glucose, the phenols were changed primarily to ethereal sulfates.

Although conjugation reactions involving glucuronic acid are well known in mammals (Harper, 1959), there is conflicting evidence in the literature regarding the abilities of fish to form glucosiduronic acids with other compounds. Brodie and Maickel (1962) reported that livers of several species, including goldfish, do not conjugate phenols with glucuronic acid in vitro, or, in vivo after intraperitoneal injections of phenol. They found that liver microsomes contained glucuronyl transerase, and phenyl glucuronids were formed when UDPGA was added to liver preparations. However, the microsomes apparently lack UDPG-dehydrogenase for conversion of UDPG to UDPGA. On the other hand, Grajcer and Idler (1963) measured conjugates of testosterone in the blood and testes of sockeye salmon. The hormone was released by the action of beta-glucuronidase, and they concluded that it had been conjugated with glucuronic acid.

The results of my investigations and those of Grajcer and Idler (1963) do not support the conclusions which Brodie and Maickel (1962) have drawn concerning the inability of fish to form glucosiduronic acids. Certainly, further research is indicated to resolve these apparently contradictory findings. Perhaps tissues other than the liver, such as the kidney, are also involved in the conjugation and excretion of Thiodan metabolites in fish.

SUMMARY

The median tolerance limits (TLm) of rainbow trout and western white suckers to Thiodan are 0.3 to 8.1 ppb, depending on temperature and exposure. Daphnia and damselfly naiads are at least seven times as resistant than fish. The naiads, in contrast to other species, appear more susceptible at colder temperatures. The effect of temperature on the toxicity of Thiodan to fish and invertebrates diminishes with exposure. Thiodan is nontoxic to trout eggs, but after hatching, the fish become increasingly susceptible with age.

The toxicity of Thiodan to suckers is not influenced by calcium or magnesium salts in the water or by mildly acidic conditions. A pH of 8.4 or 9.4 reduces or eliminates the toxicity of the insecticide withint 24 to 96 hours at 19° C. Degradation at these pH's is slower at cooler temperatures.

Investigations with carbon ^{14}C-labeled Thiodan indicate that the insecticide is taken up and deposited in various tissues of fish. The uptake is highest in liver and in the gut and feces. Rates of uptake are slower in heart, blood, gill, kidney, and brain. Western white suckers exposed to 20 ppb of labeled compound contained mean concentrations in the tissues of 1.1 to 8.4 µg per g of dry tissue. The residues in muscle and skin correlate poorly with exposure.

Goldfish are killed by daily exposures to fresh solutions containing 7 ppb of Thiodan. They succumb according to their size and the lipid content of muscle. Residues of 0.95 to 2.54 µg per g of wet tissue are detected colorimetrically in muscle. They correlate poorly with the number of treatments. Lesser amounts are found in the gonad, gill, heart, kidney, spleen, and blood, and greater levels are found in skin, liver, brain, and peritoneal fat.

Radio-tracer and chemical techniques reveal a water-soluble metabolite of Thiodan in the bile of western white suckers, northern creek chubs, and goldfish. Analyses suggest that Thiodan is degraded metabolically to its alcohol which is then conjugated with glucuronic acid and excreted via the bile into the feces.

In general, Thiodan appears useless as a selective piscicide against so-called trash

species. The chemical may be useful, however, where selectivity among fish is unimportant because: it is highly toxic to fish and less toxic to many aquatic invertebrates; it is less toxic to mammals than several other chlorinated insecticides; and it does not persist long in water of relatively high pH and temperature. The exposure of fish to Thiodan probably renders them unfit for human consumption since residues occur in edible tissues such as the muscle.

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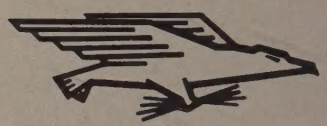

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